HUMAN MUTATION Mutation in Brief #1050, 29:E500–E519, (2008) Online

MUTATION IN BRIEF

Screening of ARHSP-TCC Patients Expands the Spectrum of *SPG11* Mutations and Includes a Large Scale Gene Deletion

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Received 6 August 2008; accepted revised manuscript 16 October 2008.

DOI: 10.1002/humu.20945

Communicated by Mireille Claustres

Autosomal recessive spastic paraplegia with thinning of corpus callosum (ARHSP-TCC) is a complex form of HSP initially described in Japan but subsequently reported to have a worldwide distribution with a particular high frequency in multiple families from the Mediterranean basin. We recently showed that ARHSP-TCC is commonly associated with mutations in *SPG11*/*KIAA1840* on chromosome 15q. We have now screened a collection of new patients mainly originating from Italy and Brazil, in order to further ascertain the spectrum of mutations in *SPG11*, enlarge the ethnic origin of *SPG11* patients, determine the relative frequency at the level of single Countries (i.e., Italy), and establish whether there is one or more common mutation. In 25 index cases we identified 32 mutations; 22 are novel, including 9 nonsense, 3 small deletions, 4 insertions, 1 in/del, 1 small duplication, 1 missense, 2 splice-site, and for the first time a large genomic rearrangement. This brings the total number of *SPG11* mutated patients in the SPATAX collection to 111 cases in 44 families and in 17 isolated cases, from 16 Countries, all assessed using homogeneous clinical criteria. While expanding the spectrum of mutations in *SPG11*, this larger series also corroborated the notion that even within apparently homogeneous population a molecular diagnosis cannot be achieved without full gene sequencing.

KEY WORDS: ARHSP; TCC; SPG11; mutation screening.

INTRODUCTION

Hereditary spastic paraplegias (HSPs), also known as Strümpell-Lorrain disease, are a heterogeneous group of inherited disorders in which the main clinical feature is progressive spasticity in the lower limbs due to pyramidal tract dysfunction (Harding 1983). Brisk tendon reflexes with bilateral Babinski sign, muscle weakness and urinary urgency are also present at clinical examination and are likely the result of a 'dying back' degeneration of the corticospinal tracts. Scholastically, HSPs are classified as pure or complex depending on whether spasticity in the lower limbs occurs in isolation or it is associated with other neurological and extraneurological signs (Harding 1983; Tallaksen et al. 2001; Depienne et al. 2007). However, an ever widening clinical spectrum, the recognition of subtle differences between apparently stereotypical neurological disorders, and the large underlying genetic heterogeneity call for a more locus-driven classification in HSP (Fink 2006; Stevanin et al. 2008b).

Autosomal recessive spastic paraplegia with thinning of the corpus callosum (ARHSP-TCC; MIM# 604360) is a frequent and complex form of HSP initially described in Japan (Nakamura et al. 1995). Since the initial reports linking Mediterranean and Asian ARHSP-TCC families to the SPG11 locus on chromosome 15q (Martinez-Murillo et al. 1999; Shibasaki et al. 2000), several groups, including our teams, proved that this phenotype has a worldwide distribution (Casali et al. 2004; Lossos et al. 2006; Olmez et al. 2006; Winner et al. 2006; França et al. 2007; Stevanin et al. 2007, 2008a) and that it is particularly prevalent in the Mediterranean basin (Casali et al. 2004; Stevanin et al. 2006). More recently, we showed that ARHSP-TCC at this locus is associated with mutations in *SPG11*/*KIAA1840* (MIM# 610844), a gene with an open reading frame of 7,787 nucleotides that comprises 40 exons and which spans a genomic region of approximately 100 Kb (Stevanin et al. 2007). *SPG11* is predicted to encode a 2,443 amino-acid-long protein, which has no homology with known proteins although a high degree of conservation is present across vertebrates. Its involvement in ARHSP-TCC was subsequently confirmed by other groups (Del Bo et al. 2007; Hehr et al. 2007; Paisan-Ruiz et al. 2008; Lee et al. 2008; Zhang et al. 2008).

We have recently reported the clinical and molecular genetic features of the largest group of *SPG11* patients, mainly coming from Western Europe and North-Africa, collected within SPATAX, an European and Mediterranean network for spinocerebellar degenerations (Stevanin et al. 2008a). We have now screened a collection of new patients referred to a single centre, and mainly originating from Italy and Brazil, in order to further ascertain the spectrum of mutations in *SPG11*, enlarge the ethnic origin of *SPG11* patients, determine the relative frequency at the level of single Countries (i.e., Italy), and establish whether there is one or more common mutation. We identified 22 new mutations and analyzed the associated phenotypes.

METHODS

Patients

Thirty-one kindred (57 patients) with an autosomal recessive inheritance and 20 isolated cases with no apparent family history of the disease were selected in our shared databases and were classified in four diagnostic categories: i) spastic paraplegia with mental retardation (MR) or cognitive impairment and thinning of the corpus callosum (TCC) demonstrated by brain MRI in at least one affected member (36 in 20 families and 8 isolated cases), ii) spastic paraplegia with TCC in at least one affected member (7 in 4 families and 10 isolated cases), iii) spastic paraplegia with cognitive impairment but without TCC in all affected members (7 in 4 families and 2 isolated cases), iv) "apparently" pure spastic paraplegia without TCC at MRI (7 cases in 3 families).

Clinical and paraclinical evaluations followed a protocol established by the SPATAX network (coordinator: Dr A. Durr) that included: full medical history and physical examination, estimation of the age and the symptoms at onset in the patient, disease duration and severity according to the Spastic Paraplegia Rating scale (Schule et al. 2006), presence/absence of additional neurological symptoms/signs, electromyographic (EMG) and nerve conduction velocity (NCV) studies, and brain and spinal cord MRI whenever possible. Neuropsychological evaluations were performed in the vast majority of cases by measuring their IQ, the Mini Mental State Evaluation (MMSE), and the Wechsler Memory Scale (Wechsler 1987). According to the DSM-IV criteria (2000), mental retardation was considered when the patient had an IQ$\leq$ 70 before the age of 18 years.

Having received the patients' (or their parents') written informed consent and the authorization of our local Ethical Committees, we purified genomic DNA from peripheral blood samples for subsequent genetic analyses. In all the patients, we had previously ruled out other causes of acquired spastic paraplegia such as multiple sclerosis, adrenoleukodystrophy, and mitochondrial diseases, as described (Casali et al. 2004).

Fifteen of the 31 families were Italian, eight were Brazilian, two were Turkish and one each was from Malta, Germany (with Turkish origin), Austria, The Netherlands, Montenegro, and Slovenia. Seventeen families were non-consanguineous and 14 consanguineous. Most of the 20 isolated patients were Italian (n=14), three Brazilian whereas the remaining originated from other European countries. In sporadic cases, mutations or rearrangements in the *SPG4* gene and mutations in the *SPG7* and *SPG21* genes had also been excluded (data not shown).

Mutation detection

The coding sequence and splice site boundaries of the 40 exons of the *SPG11*/*KIAA1840* gene (GenBank reference sequence NM_025137.3) were amplified by PCR and sequenced on both strands using the Big Dye chemistry (Applied Biosystems, Foster City, CA) as previously described (Stevanin et al. 2007). In non mutated cases, in addition to the coding sequence, 1166 bp upstream the first ATG and 425 bp downstream the last termination codon of the *SPG11* gene were amplified with a classical protocol and sequenced using four primers pairs (Supp. Table S1). The sequence products were run on an automated ABI 3730 sequencer and the results were analyzed with SeqScape 2.5 software (Applied Biosystems). Alternatively, PCR products generated using primers and PCR conditions indicated in Supp. Table S2 were processed for denaturing High Performance Liquid Chromatography (d-HPLC). To this end, the untreated PCR products (5–15µl) from the patients and a control DNA — previously deemed free of sequence variants — were mixed, denatured at 94°C for 5 minutes and renaturated overnight at room temperature to allow the formation of heteroduplex prior to d-HPLC analysis. Samples were then analyzed using the Transgenomic WAVE DHPLC system (Transgenomic Inc, Omaha, NE) and results were interpreted essentially as reported (Patrono et al. 2005).

Segregation of mutations detected in this study was verified by direct sequencing or d-HPLC, or both in all available family members whenever possible. In addition, the relative frequencies of missense variants were determined on unrelated healthy controls (100 Italians and 50 European Caucasians), for a total of 300 chromosomes, by d-HPLC, ad hoc designed PCR-restriction fragment length polymorphisms (data not shown) or direct sequencing. Synonymous, missense and splice site variations were systematically evaluated for modifications of exonic splicing enhancers (www.rulai.cshl.edu/cgi-bin/tools/ESE/esefinder.cgi) or consensus splicing sequences in order to determine the splice site score

(rulai.cshl.edu/new_alt_exon_db2/HTML/score.html and www.fruitfly.org/seq_tools/splice.html). Multiple alignments with spatacsin orthologs were performed using ClustalW (www.ebi.ac.uk/clustalw/) to evaluate the degree of conservation of missense variants.

Nomenclature of mutations and variants followed the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen) and referred to the cDNA sequence (GenBank reference sequence version NM_025137.3) with the A of the translation initiation codon as +1.

Haplotyping

In order to identify possible shared haplotypes between families carrying the same mutations, we genotyped the *SPG11* flanking microsatellites *D15S781, D15S537, D15S516*, and *D15S659*. We also used two new flanking markers, one of which lies in *SPG11* (primers listed in Supp. Table S1). PCR-amplified fragments were pooled with GeneScan500Liz marker, sized on an ABI 3730 automated sequencer and analyzed with GeneMapper 4 software (Applied Biosystems), according to the manufacturer's recommendations. Haplotypes were manually reconstructed considering the minimal number of recombinations.

Other methods

Cultured skin fibroblast polyA+ RNA was purified and reversely transcribed using the 1st Strand cDNA Synthesis Kit (Roche, Hamburg, Germany) according to the manufacturer's random primer protocol, and the consequences of the exon 24 c.4046T>A/p.F1349I mutation on splicing in patient F33[COL] was examined by RT-PCR using primers designed in exons 16 and 30.

To determine the segregation of the large-scale rearrangement in family F-NL02 we PCR-amplified a genomic fragment using oligonucleotide primers Spg11-30delF (5'-3') AAT GTA TTG GGT TGC TTT CCT G and Spg11-35R (5'-3') CCC TCC ATT TTC CCA AGA GT, High Fidelity Taq Polymerase (Roche Diagnostic, Milan, Italy) and PCR conditions consisting of 25 cycles of 15 s at 94°C, 30 s at 57°C, and 8 min at 68°C with an increase of 5 s/cycle after the first 10 cycles.

RESULTS

SPG11 mutation screening

SPG11 mutation screening has been undertaken in at least one index case in 31 families with a neurological picture highly resembling the clinical features of ARHSP-TCC (Shibasaki et al. 2000; Casali et al. 2004; Lossos et al. 2006; Stevanin et al. 2006; Winner et al. 2006) and in whom linkage to chromosome 15q had not been established *a priori*. We also included in the study a subset of isolated cases (n=20). In a total of 25 of these patients (16 familial index cases and 9 sporadic), we detected 32 different sequence changes in the coding regions or consensus splice sites of the *SPG11* gene; 22 of these are novel mutations whereas 10 have previously been reported (Table 1).

Table 1. List of *SPG11* mutations identified in this study (GenBank reference sequence NM_025137.3).

Mutation Type	Nucleotide/Amino acid position	Exon	Homo- or heterozygous state	Family (n index cases)	Origin	Reference
Nonsense	**c.268G>T/p.E90X**	**2**	**Heterozygous**	**F-BRA12175 (1)**	**BR**	**This study**
	c.1679C>G/p.S560X	**8**	**Heterozygous**	**F16[SB] (1)**	**IT**	**This study**
	c.1951C>T/p.R651X	**10**	**Heterozygous**	**MP; F28[VAC] (3)**	**IT**	**This study**
	c.2697G>A/p.W899X	**15**	**Homozygous**	**F10; TK-SH (4)**	**TR**	**This study**
	c.5470C>T/p.R1824X	**30**	**Heterozygous**	**F8 (1)**	**DE**	**This study**
	c.5870C>G/p.S1957X	**31**	**Heterozygous**	**F16[SB] (1)**	**IT**	**This study**
	c.5970C>G/p.Y1990X	**31**	**Homozygous**	**FA (2)**	**IT**	**This study**
	c.6091C>T/p.R2031X	32	Homozygous	F1 (3)	TR/DE	Lee et al. 2008
	c.6100C>T/p.R2034X	32	Homozygous	F-BRA21325 (2)	BR	Stevanin et al. 2007
	c.6856C>T/p.R2286X	**38**	**Heterozygous**	**F14[CAL] (1)**	**IT**	**This study**
	c.7023C>A/p.Y2341X	**39**	**Homozygous**	**F-BRA21215 (2)**	**BR**	**This study**
Insertion/deletion	**c.408_428del21/p.E136_I142del**	**2**	**Heterozygous**	**F35[TER] (1)**	**IT**	**This study**
	c.529_533del5/p.I177_F178del>S177fsX180	3	Heterozygous	F17[BRA]; F-BRA12175 (2)	BR	Stevanin et al. 2007
	c.733_734del2/p.M245VfsX247	4	Hetero- and Homozygous	F-BRA12162; F-BRA19070; F35[TER] (5)	BR; IT	Stevanin et al. 2007
	c.1203delA/p.K401fsX415	6	Heterozygous	F17[SP]; FB (3)	IT	Stevanin et al. 2007
	c.1697_1712del16insTACTCCCAT/p.D566VfsX595	**8**	**Heterozygous**	**DKD (2)**	**IT**	**This study**
	c.2355_2356del2/p.K785SfsX796	**13**	**Homozygous**	**F34[GARG] (1)**	**IT**	**This study**
	c.2716delC/p.Q906SfsX920	**15**	**Homozygous**	**F9 (2)**	**BR**	**This study**
	c.2849_2850insT/p.L950FfsX953	**16**	**Heterozygous**	**MP (2)**	**IT**	**This study**
	c.3075_3076insA/p.E1026RfsX1029	17	Heterozygous	F8; F33[COL] (2)	DE; IT	Hehr et al. 2007
	c.3664_3665insT/p.K1222IfsX1236	**21**	**Heterozygous**	**F17[SP] (1)**	**IT**	**This study**
	c.3741_3742insA/p.P1248TfsX1264	**22**	**Heterozygous**	**F17[BRA] (1)**	**BR**	**This study**
	c.4307_4308del2/p.Q1436RfsX1442	25	Heterozygous	F16[BRA] (1)	BR	Stevanin et al. 2008a
	c.5255delT/p.F1752SfsX1837	30	Heterozygous	SP (2)	IT	Hehr et al. 2007
	c.5987_5990dupCTCT/p.C1996fsX1999	**31**	**Heterozygous**	**DKD (2)**	**IT**	**This study**
	c.5986_5987insT/p.C1996LfsX1999	31	Heterozygous	SP (2)	IT	Stevanin et al. 2008a
	c.5992insT/p.Y1998fsX1999	**31**	**Heterozygous**	**FB (2)**	**IT**	**This study**
	c.5898+5493_6509-491del/p.T1966fsX1968	**31-34**	**Homozygous**	**F-NL02 (1)**	**NL**	**This study**
Splice-site	**c.2444G>T/p.R815M, r.?**	**13**	**Heterozygous**	**F28[VAC] (1)**	**IT**	**This study**
	c.2444+1G>C, r.?	**Intron 13**	**Heterozygous**	**F16[BRA] (1)**	**BR**	**This study**
	c.2833A>G/p.R945GfsX950, r.2834_2835ins2834+1_2834+65	15	Heterozygous	F14[CAL] (1)	IT	Stevanin et al. 2008a
Missense	**c.4046T>A/p.F1349I**	**24**	**Heterozygous**	**F33[COL] (1)**	**IT**	**This study**

New mutations are indicated in bold. Origin of patients adopts the ISO indication for Country code.

Figure 1 shows a sample of the mutations detected in this study. In 24 cases, including 9 sporadic patients, the segregation was verified within the family throughout the analysis of 60 additional samples, of whom 15 were affected and 45 healthy relatives. Overall, 40 patients showed to have *SPG11* mutations on both alleles.

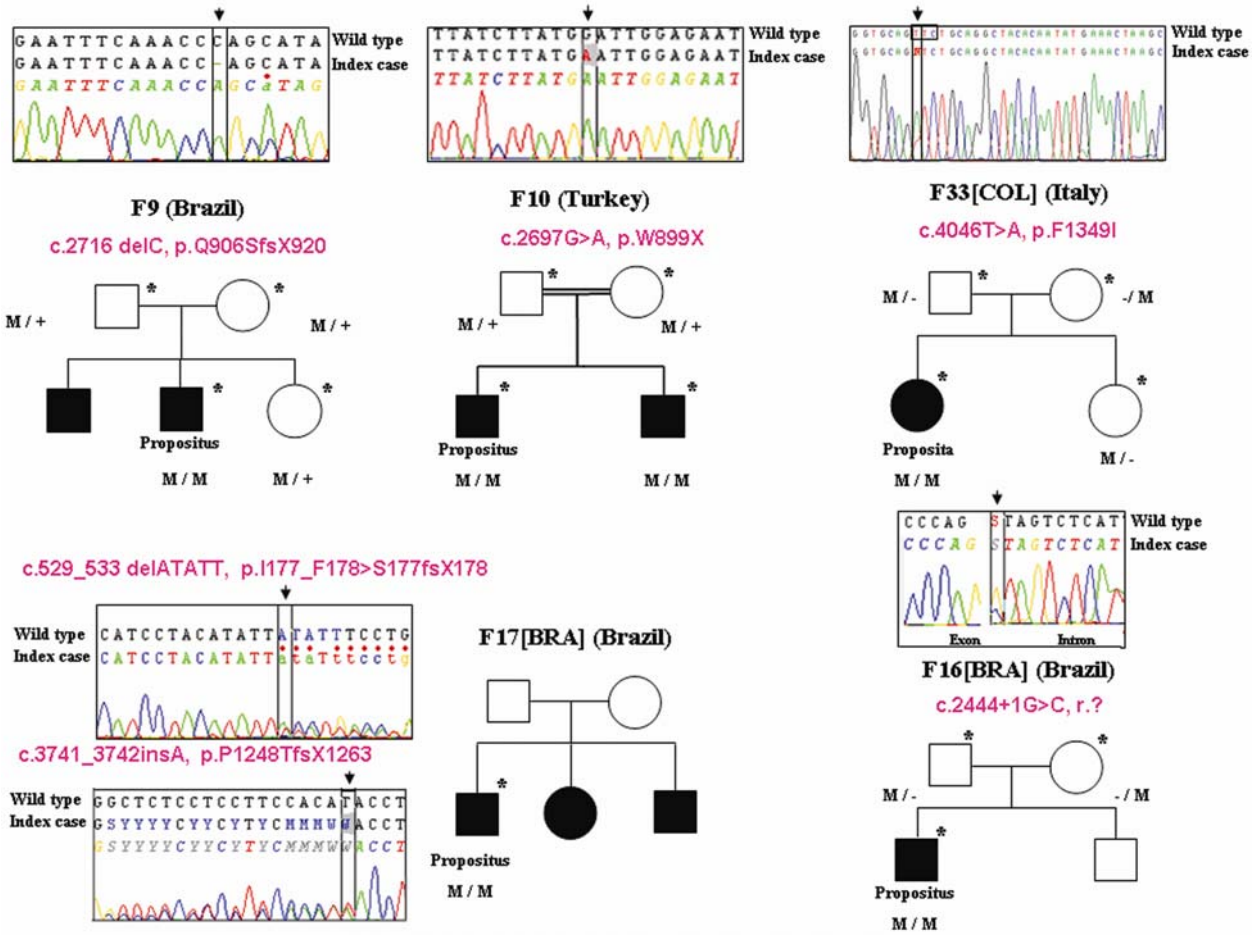

Figure 1. A sample of the new SPG11 mutations identified in this study. A selection of the electropherograms flanking novel mutations in the SPG11 gene is shown. Their segregation in the patients' family is also indicated. The novel mutations are indicated by arrows. Square symbols are men, the circles are women. The filled symbols are affected individuals. Stars indicate sampled subjects. M = mutation; + = wild type.

The index cases in 12 kindred presented with a homozygous mutation, whereas two heterozygous variants were detected in four families. Not too surprisingly, we only found compound heterozygosity in the 9 remaining isolated cases, all non-consanguineous. The majority of the mutations (Table 1) were predicted to be pathogenic because they lead to an early stop codon or frameshift, producing a premature termination of translation, and included nonsense mutations (n = 11), small deletions (= 8), small insertions (= 6), small duplication (= 1). It is of note that in one family (DKD) we detected a new micro-rearrangement (c.1697_1712del16insTACTCCCAT/p.D566VfsX595) at the compound heterozygous state. Also, we detected the first homozygous large-scale rearrangement sized about 9 Kb (c.5898+5493_6509-491del) in a Dutch family (F-NL02) predictably resulting in premature protein truncation at residue 1968 (p.T1966fsX1968) (Figure 2). The mutation deleted the sequences of exons 31-34 and was flanked by a 42-bp repeat sequence (5'-ggtggctcacgcctgtaatcccagcactttgggaggccgagg -3'). Moreover, three changes (c.2444G>T; c.2444+1G>C; c.2833A>G) – which were ruled out in 150 controls and expected to involve nucleotides predicted to alter the correct splicing of the *SPG11* mRNA — were detected on one allele in three families. In the Italian family F28[VAC] the c.2444G>T mutation affected the last nucleotide of exon 13, predicting

a missense change (p.R815M), but also altering the 5'-splicing donor site of intron 13 (splice score of +0.2 versus +3.7). In the Brazilian kindred F16[BRA], mutation c.2444+1G>C affected the first base of the splice donor site and likely led to abolition of splicing at this site (splice score of -7 versus +3.7). Although strongly predicted *in silico*, effects on splicing could not be experimentally tested in living cells. In addition, the c.2833A>G variation detected in the Italian family F14[CAL] affected the last nucleotide of exon 15, predicted a missense change (p.R945G) and caused the reduction of the splice score from +4.9, for the wild type, to +2.7. When this same mutation was detected in an ARHSP-TCC Israeli kindred (Stevanin et al. 2008a), analyses of mRNA from patients have shown alteration of *SPG11* mRNA splicing with retention of 65-bp of intron 15 and subsequent early stop codon (r.2834+1_2834+65ins/ p.R945GfsX950). Interestingly, a missense mutation was detected in a single Italian patient F33[COL] who harbored the c.4046T>A/p.F1349I mutation in compound heterozygosity with the c.3076_3077insA/p.E1026RfsX1029. Residue F1349 is a well-conserved amino acid in other vertebrates and was not found mutated in 300 control chromosomes of European ancestry. When tested in cultured skin fibroblasts from the patient, the c.4046T>A/p.F1349I did not affect mRNA stability or correct splicing (not shown).

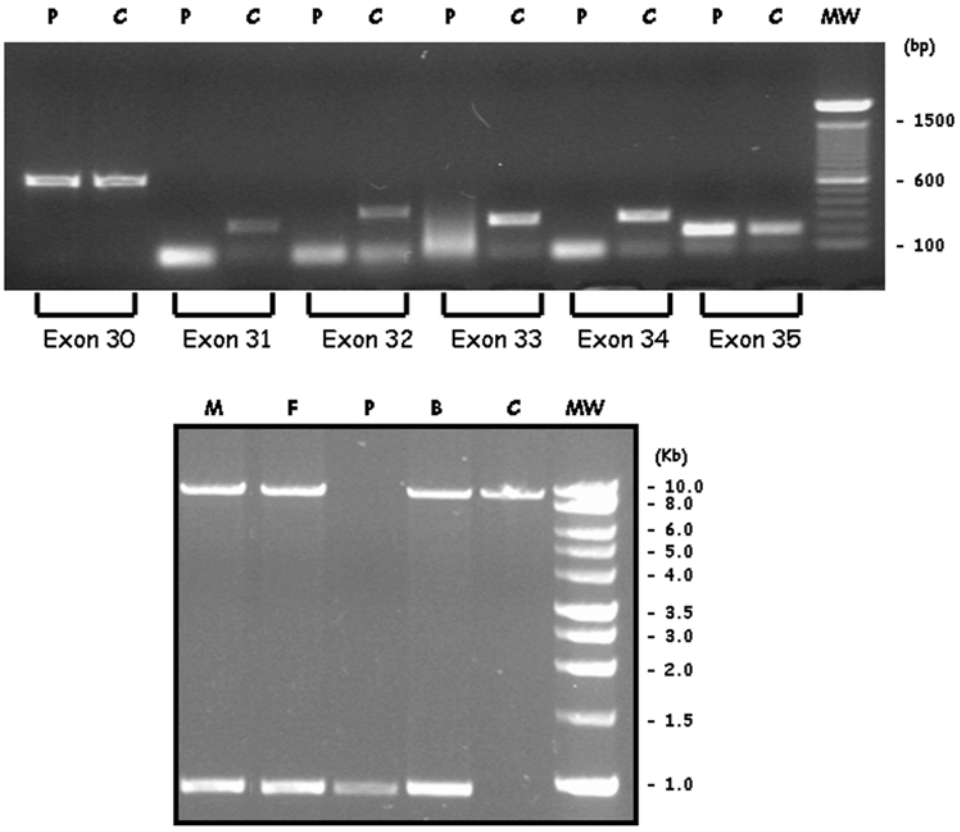

Figure 2. A novel c.5898+5493_6509-491del large-scale rearrangement encompassing exons 31-34 of the SPG11 gene. Top panel. Polymerase Chain Reaction (PCR) detection of the novel c.5898+5493_6509-491del large-scale rearrangement in SPG11 found in family F-NL02. C, control; P, patient F-NL02; MW, DNA 100-bp molecular marker size. Bottom panel. Using PCR conditions outlined in the text (see methods), a ~9.0 Kb fragment is amplified in a normal control (C) whereas a 720-bp fragment is detected in the propositus of kindred F-NL02 (P) as the result of the homozygous large scale deletion. Both the healthy parents (F, father and M, mother) and the brother (B) carried the heterozygous deletion. MW, DNA 1-Kb molecular size marker.

Molecular analyses identified families harboring the same mutation and sharing the same haplotype although they bear different surnames and formally deny relationships. As an example, we detected the homozygous c.2697G>A (p.W899X) in two presumably unrelated Turkish families (F10 and TK-SH) and the homozygous c.733_734del2 (p.M245VfsX247) variant in two seemingly unrelated Brazilian kindred (F-BRA12162 and F-BRA19070) (see

Table 2). However, we cannot exclude the existence of a distant common ancestor. The c.733_734del2 mutation has already been reported in four families, two of which are shown in Table 2, in association with different haplotypes (Stevanin et al. 2007; Del Bo et al. 2007; Hehr et al. 2007; Stevanin et al. 2008a). Moreover, we found the c.1951C>T (p.R651X) in two apparently unrelated families who originated from the same geographical region in central Italy and shared partial common haplotype, although different from the previously reported patient from Romania (Stevanin et al. 2008a). We also observed mutations recurring in several ethnicities. As an example, we found the frequent homozygous c.6100C>T (p.R2034X), already detected in northern African families (Stevanin et al. 2007; Stevanin et al. 2008a; Boukhris et al. 2008a), in one Brazilian kindred with partially common haplotypes (see Table 2). Similarly, the c.529_533del5 (p.I177_F178del>S177fsX) mutation, already reported in three Portuguese families (Stevanin et al. 2007; Stevanin et al. 2008a), was also detected in an additional Brazilian family again segregating with a similar disease-bearing chromosome.

Besides pathogenic changes in *SPG11*, we detected two reported homozygous SNPs (dbSNP: rs3759875 and rs3759873), two frequent heterozygous polymorphisms (p.F463S, and p.Y2341Y) (Stevanin et al. 2008a), and one new variant (p.K1273R); these variants were also detected in about 3% of normal alleles.

No mutations in coding exons and flanking introns were detected in 15 familial index cases, and in 11 isolated patients. Sequencing of 1166 bp upstream the first ATG and 425 bp downstream the last termination codon did not reveal mutations, too. These cases were not informative enough to formally exclude the involvement of *SPG11* through microsatellite genotyping, however.

Clinical characteristics of the new *SPG11* patients

Clinical information was available for all cases in which we could ascertain the presence of two mutant alleles. Table 3 reports clinical findings in the 40 individuals who proved to carry pathogenic variants on both alleles.

Familial and sporadic cases did not present with distinguishable clinical profiles. Also, the phenotype of patients harboring the missense mutation was similar to that of the patients having variants predicting early protein truncation. The disease followed a broadly similar course and affected siblings tended to show the same pattern of symptom evolution and rate of disease progression. Age at onset of the disease ranged from 5 to 23 with a mean of 14.0±4.1 years and it was mostly characterized by difficult walking (75%) with leg stiffness, gait abnormalities and in some cases frequent falls whereas learning disabilities were the presenting symptom in 13% of the cases. In the remaining cases, both deterioration of gait and cognitive delay occurred at the same time or their onset could not be recorded accurately. In a single patient (DKD-H83) the disease manifested initially with upper limbs tremor at age 17 years but pyramidal tract signs and cognitive delay followed within two years. After a mean disease duration of 7.9±5.5 years, all affected members of the families presented with a mild to severe spasticity of the legs with weakness in most. Deep tendon reflexes were brisk in the legs and, after less than 10 years, also in the arms. Most of the patients had mental impairment (87%) with objective evidence in 20 patients with IQ ranging from 53 to 69. Few cases initially showed a low-normal IQ but scored poorly at subsequent testing.

The MRI scans were not recorded in three cases with disease duration of two, four and 12 years. However, their similarly affected sibs had shown typical neuroimaging features of TCC. Neuroradiological evidence of TCC was present in 34/37 (91.8%) patients with scans recorded whereas white matter hyperintensities were detected in 57%. Cortical or cerebellar atrophy, or both, were noted in seven patients (19%). Two familial cases only presented with moderate white matter signal abnormalities at the frontal horns of the lateral ventricles. Only one patient had a normal MRI, but after a disease duration of less than one year.

Less frequent additional neurological findings consisted of cerebellar ataxia in 32% and extrapyramidal features in 16% of the cases (Table 3), usually in late stages of the disease. Signs of axonal neuropathy were detected in 15/34 patients (44%). Few patients showed neurogenic changes resembling lower motor neuron involvement. A slow electroencephalogram was also recorded in two cases. A single patient showed reduced visual acuity at night but neither electroretinogram nor detailed fundoscopy were performed; another case carried a psychiatric diagnosis of bipolar disorder with psychosis and hallucinations. Concurrent Down syndrome was also observed in one case (Table 3).

Table 2. Haplotypes of six close markers segregating with the recurrent mutations in the *SPG11* gene in this study and in previous reports.

Family Origin	F10 Turkey	TK-SH Turkey
D15S781	185	185
D15S537	180	180
*Chr15 :42720928^*	280	280
SPG11 exon 15	*c.2697G>A*	*c.2697G>A*
Chr15 :42800751¤	198	198
D15S516	195	195
D15S659	199	199

Family Origin	F-BRA12162 Brazil	F-BRA19070 Brazil	FSP117* France	FSP870# Tunisia
D15S781	187	187	185	187
D15S537	160	160	172	180
SPG11 exon 4	*c.733_734del2*	*c.733_734de2*	*c.733_734del2*	*c.733_734del2*
*Chr15 :42720928^*	272	272	278/280	286
Chr15 :42800751¤	198	198	196/198	196
D15S516	195	195	195	195
D15S659	175	179	179	195

Family Origin	F28[VAC] Italy	MP Italy	FSP683# Romania
D15S781	185	185	187/191
D15S537	172/176	172/176	164/184
*Chr15 :42720928^*	278/280	278/280	278/280
SPG11 exon 10	*c.1951C>G*	*c.1951C>G*	*c.1951C>G*
Chr15 :42800751¤	198	198	194/198
D15S516	195	195	193/195
D15S659	199/179	195/183	199/179

Family Origin	FSP732* Algeria	FSP446* Morocco	FSP881# Tunisia	FSP221* Algeria	FSP792# Morocco	FS845# Morocco	F-BRA21325 Brazil	FSP400# Algeria
D15S781	185	185	185	185	185	185	185	185
D15S537	176	180	180	176	176	176	176	180
*Chr15 :42720928^*	282	280	280	280	280	280	280	280
SPG11 exon 32	*c.6100C>T*	*c.6100C>T*	*c.6100C>T*	*c.6100C>T*	*c.6100C>T*	*c.6100C>T*	*c.6100C>T*	*c.6100C>T*
Chr15 :42800751¤	198	198	198	198	196	196	196	196
D15S516	191	191	191	191	191	191	191	191
D15S659	175	179	179	179	179	179	191	195

Family Origin	FSP831# Portugal	FSP754* Portugal	FSP386* Portugal	F17[BRA] Brazil	FSP386* Portugal
D15S781	185	185	185	185	185
D15S537	172	172	172	ND	172
SPG11 exon 3	*c.529_533del5*	*c.529_533del5*	*c.529_533del5*	*c.529_533del5*	*c.529_533del5*
*Chr15 :42720928^*	286	286	286	282/286	286
Chr15 :42800751¤	196	196	196	196	194
D15S516	195	195	195	195	193
D15S659	203	203	195	195	195

*Stevanin et al. 2007; Del Bo et al. 2007, #Stevanin et al. 2008a, ND= not done; ^ = Intragenic marker; ¤ = 3' UTR marker. Conserved regions are highlighted in grey. Genotypes are indicated in base pairs.

Table 3. Clinical features of 40 patients with mutations in the *SPG11* gene.

M= male, F= female, ND= not done, UL= upper limbs, LL= lower limbs, IQ= intellectual quotient, MMSE= Mini Mental State Evaluation, ++= increased; += present; -= absent; PWM, periventricular white matter; TCC= thin corpus callosum.

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F-BRA12175	BR	12/F	7	Difficulties at school	Mild	Mild/No	+	++	MUTE	-	Delay	TCC, PWM	Normal	
F16[SB]	IT	25	10	Difficulties in concentration and walking	Moderate	Moderate spasticity, Walking difficulties	++	+++	+	+	Mental deterioration	TCC, PWM	Neuropathic changes	Slight tremor finger to nose
F17[BRA]	BR	17/F	15	Spasticity	Mild	Mild/No	+	+++	+	-	Retarded	TCC, PWM	Normal	
MP-1	IT	26/M	18	Spasticity	Severe	Severe/ Moderate	Normal	++	++	-	Mental retardartion/ Progressive cognitive deterioration	TCC, PWM, Cortical atrophy	Axonal Neuropathy	
MP-2	IT	21/F	20	Spasticity	Moderate	Moderate/ Moderate	++	++	++	-	Moderate	TCC, PWM	Axonal Neuropathy	Urinary urgency
F28[VAC]	IT	18F	14	Feet deformities and stiff legs	Moderate	Moderate/ Mild	-	++	+	-	No cognitive deficit	TCC, PWM	Normal	Severe pes cavus
F10-1	TK	12/M	10	Falls	Severe	Severe/ Moderate	++	++	++	+	Mental retardation/ Progressive cognitive deterioration	TCC, PWM, Cortical atrophy	Axonal Neuropathy	
F10-2	TK	16/M	14	Tremor	Moderate	Severe/ Moderate	+	++	+	-	Cognitive delay	ND	ND	Slow EEG
TK-SH-1	TK	27	8	Deterioration of gait	Moderate, at 22 yrs still able to walk with crutches	Moderate LLspasticity, Feet drop due to weakness of ant. tibial muscle	++	+++	++	+	Some cognitive decline	Mildly increased signal of periventricular and deep cerebral white matter, “Flames” at the frontal horns, Mild cerebral atrophy, TCC	Axonal Neuropathy	Dysartria, Decreased manual dexterity
TK-SH-2	TK	22	10	Deterioration of gait	Mostly wheelchair dependent	Moderate LLspasticity, Feet drop due to weakness of tibial muscle	++	+++	Equivocal	+	Cognitive decline	ibidem	Axonal Neuropathy (mild)	Down syndrome, Progressive serious dysarthria, Decreased manual dexterity

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F8	DE	24/F	10	Deterioration in school performance (10yrs), Gait disturbance (14 yrs)	Progressive deterioration, now severely disabled	Standing with support, No walking	+++	+++	+	-	Initial normal cognitive development, Mental deterioration starting at 10 yrs	TCC, PWM, Cortical atrophy	NC studies normal, EMG of ant. tibial muscle showed mild neurogenic changes at 17 yrs	Foot drop
FA-1	IT	17/F	15	Spasticity	Mild	Moderate/ No	+	+++	++	-	Mental retardation, Progressive cognitive deterioration	TCC	ND	Visual problems at night
FA-2	IT	12/F	12	Delayed	Mild	Mild/No	+	++	++	-	Delayed learning and memory	Normal	ND	
F1-1	TK-DE	22/F	10	Walking difficulties	Moderately impaired	+++	++	+++	+	-	Severe, she can hardly add 4 + 5	TCC	ND	
F1-2	TK-DE	24/M	15	Walking difficulties	Now: can hardly walk	+++	++	+++	-	-	Now communication via language is hardly possible, but he can add 4 + 5	TCC	Nerve conduct. study compatible with mild axonal loss	Nerve biopsy 2000: mild axonal loss, CK mildly elevated
F1-3	TK-DE	17/F	16	Pain in her legs	Gait only mildly impaired	+	+	+++	+	-	Mildly impaired	TCC	Normal NCV, Normal MAP-amplitudes, Normal needle EMG	CK normal
F-BRA21325	BR	21/F	17	Walking difficulties	Use of walker	Severe	++	+++	+	-	Mental retardation	TCC, PWM	Normal	
F-BRA21326	BR	18/M	15	Walking difficulties	Moderately impaired	Moderate	++	+++	+	-	Mental retardation	TCC	Normal	
F14[CAL]	IT	29/F	17	Hypostenia, Difficulties in walking	Severe	Severe, unable to walk unaided	+++	+++	+	+	Mental deterioration	TCC, PWM	Axonal Neuropathy	
F-BRA21215	BR	27/F	15	Gait abnormalities	Wheelchair at 24 yrs	Severe	+	+++	MUTE	-	Mental retardation	TCC	Normal	Dysarthria
F-BRA21216	BR	21/F	17	Gait abnormalities	Moderate	++	+	+++	+	-	Mental retardation	TCC	ND	Pes cavus
F35[TER]	IT	46	15	Spasticity	Wheelchair at 23 yrs	Severe	+	+++	+	+	Mental retardation	TCC	Neurogenic	Severe psychosis, Allucinations

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F-BRA12162	BR	24/M	8	Deterioration in school performance	Deterioration, now severely disabled	Standing with support, no walking	+++	+++	+	-	Mental retardation	TCC	ND	
F-BRA12163	BR	22/F	10	Walking difficulties	Moderately impaired	+++	++	+++	+	-	Mental retardation	ND	ND	
F-BRA19070	BR	32/M	16	Spasticity	Wheelchair at 27 yrs	Severe	-	+++	+	-	Mental retardation	TCC	Normal	
F-BRA19069	BR	33/F	22	Stiff legs	Cane at 32	Moderate	-	+++	+	-	Mental retardation	TCC	ND	
F-BRA19271	BR	27/F	23	Gait abnormalities	Cane at 26	Moderate	-	+++	+	-	Mental retardation	ND	ND	
F17[SP]	IT	29/M	6	Mild learning disabilities	Mild	Moderate	+++	+++	+	-	Mental retardation/ Progressive cognitive deterioration	TCC, PWM	Normal	Urinary urgency
FB-1	IT	28/F	17	Feet deformities and stiff legs	Severe	Moderate/ Mild	+	-	++	+	Mental retardation/ Progressive cognitive deterioration	TCC, Cerebellar atrophy, Cortical atrophy	Axonal Neuropathy	Dysarthria, Severe pes cavus
FB-2	IT	24/F	19	Falls	Severe	Moderate/ Mild	+	-	++	-	Mental retardation/ Progressive cognitive deterioration	TCC	Axonal Neuropathy	
DKD-H82	IT	30	13	Stiffness while walking	Severe	Marked/severe unable to walk	+++	+++	+	+	Mental deterioration (2007 severe)	Striking TCC, PWC	Neurogenic changes	
DKD-H83	IT	26	17	Hand tremor and difficulties in walking	Moderate	Marked walking difficulties	+++	++	+	+	Mental deterioration	ibidem	Neurogenic changes	Slight adiadokinesia at finger to nose test
F34[GARG]	IT	29/F	22	Spasticity	Mild	Moderate	-	++	+	-	-	PWM	Normal	
F9-1	BR	22/M	14	Gait disturbances	Severe	Severe/ Moderate	+	++	++	-	Mild decline	TCC	Normal	Slow eeg, Dysathria
F9-2	BR	20/M	12	Pain in LL, Gait disturbances	Severe	Severe/ Moderate	++	++	++	-	Mild decline	TCC	ND	Pes cavus, Tremor upper limbs
F33[COL]	IT	27/F	15	Learning slow	Mild	Moderate	+	++	+	-	Cognitive delay	TCC, PWM	Neurogenic	Dysarthria
F16[BRA]	BR	23/F	17	Stiff legs	Mild	++	+	+++	+	-	Mental deterioration	TCC, PWM	Normal	Dysarthria
SP-H28	IT	20/F	15	Learning difficulties	Moderate	+++	++	+++	+	+ (distal)	Mild decline	TCC, PWM	Normal	Pes varus, Fragmented smooth pursuit
SP-H29	IT	13/M	12	Walking difficulties	Moderate	++	+	-	+	-	-	PWM	Axonal Neuropathy	Pes varus, Fragmented smooth pursuit

Patient ID code	Origin (ISO cod)	Age at examination (years)/ sex	Age at onset (years)	Symptom at onset	Severity	LL spasticity/ weakness	LL reflexes	UL reflexes	Ext plantar	Muscle wasting	Mental Retardation/ Cognitive decline	MRI	EMG	Other
F-NL02	NL	15	5	Deterioration of gait	Moderate, at 15yrs still able to walk	Moderate LL spasticity	++	+++	++	-	-	Mildly increased signal of periventricular and deep cerebral WM, “Flames” at the frontal horns, Mild cerebral atrophy, Thin anterior CC	Normal	Mild Dysarthria

DISCUSSION

In a large cohort of 31 families with HSP-TCC and in 20 sporadic cases we identified *SPG11* disease alleles in 25/51 index patients (49%). Broadening the spectrum of mutations in spatacsin, we identified 32 variations, of which 22 are novel, distributed all over the gene. Eleven nonsense mutations, eight small deletions, six small insertions, one small duplication and three mutations with effects on splicing were identified. Moreover, we detected one in/del and one large scale deletion sized about 9.0 Kb. The causative nature of the missense p.F1349I variant remains to be proved because single heterozygous deletions can have escaped detection in the carrier patient. Nonetheless, the p.F1349I, which affects a conserved amino acid and was associated with a truncated allele in trans, was absent in 300 control chromosomes. Moreover, this mutation is predicted to be intolerant (PolyPhen — www.genetics.bwh.harvard.edu/pph/ — and SIFT www.blocks.fhcrc.org/sift/SIFT.html) and could lead to loss of function of the protein.

Together with the already reported changes (Stevanin, et al. 2007; Del Bo et al. 2007; Hehr et al. 2007; Stevanin, et al. 2008; Boukhris et al. 2008a; Paisan-Ruiz et al. 2008; Zhang et al.2008; Lee et al. 2008; Erichsen et al. 2008), disease-associated mutations in *SPG11* are now 67 and almost invariably alter the correct formation of a complete protein product in all except one case. If formed, the protein would be rapidly degraded or significantly shortened. Consequently, no significant domain-related clinical differences could be observed. As an example, the c.529_533del5 mutation lies in the first transmembrane domain whereas the c.3741_3742insA in the third transmembrane domain and the large deletion removes the Myb domain. These mutations presented with comparable age at onset, disease severity and progressive neurological impairment. The practical consequence of the large number of different mutations identified, in terms of the diagnosis of ARHSP-TCC, is that a quick, DNA-based test seems unfeasible. We can speculate that a protein-based diagnostic assay of the type being developed in other forms of spinocerebellar degenerations [i.e., *ALS2* (Eymard-Pierre et al. 2006), ataxia-telangectasia syndrome (Sutton at al. 2004) or ataxia with oculomotor apraxia type 1 (Ferrarini et al. 2007)] will be more effective.

Our study enlarges the ethnic origin of *SPG11* patients. Like in previous studies (Hehr et al. 2007; Stevanin et al. 2008a), no population-related mutation seems to emerge in both Italian and Brazilian cases and frequent compound heterozygosity is observed which in turn might imply higher than expected carrier frequencies. Even within apparently homogeneous populations, a molecular diagnosis cannot be achieved without full gene sequencing. For example, in a total of 29 Italian index cases with compatible phenotypes (15 familial and 14 apparently sporadic patients), we detected 12 subjects — half of whom had no positive family history — harboring pathogenic variants in *SPG11* (41%) and 21 different mutations broadly distributed along the coding exons. A similar ample allelic heterogeneity has been reported in Tunisian (Boukhris et al. 2008a), Pakistani (Paisan-Ruiz et al. 2008) and Turkish families (Hehr et al 2007). Interestingly, few mutations seem to be recurrent in patients' population originating from the Mediterranean basin, indicating founder effects with subsequent migrations. The haplotypes segregating with the c.529_533del5 *SPG11* mutation are particularly well conserved in the Brazilian and Portuguese cases, but the telomeric and centromeric portions of the haplotype segregating with the c.6100C>T mutation in patients from North Africa and Brazil are more divergent and might indicate a more ancient mutational event. However, a mutational hot spot cannot be excluded. Additional mutated families are required to date these events.

When clinical features are driving molecular testing, roughly one case in two of familial complicated spastic paraplegia harbors *SPG11* mutations (Table 4). Although we (Stevanin et al. 2007; Stevanin et al. 2008a; Boukhris et al. 2008a, and this study) and others (Hehr et al. 2007; Paisan-Ruiz et al. 2008) have at times noticed that intrafamilial differences are possible, and similar mutations not necessarily lead to similar onset and disease course (Table 3), *SPG11* patients usually develop a progressive motor and cognitive deterioration from school years, but earlier learning problems and attention deficits are likely to occur. Combining evidence from this study and our previous reports (Stevanin et al. 2007; Stevanin et al. 2008a) it is clear that the presence of motor deterioration with signs of pyramidal dysfunction of the legs in young students who perform badly in junior-high school appears sufficient to propose a molecular testing after appropriate clinical and imaging evaluation. Practically all *SPG11* mutations are associated with a widely similar disease course with frank spastic paraplegia manifesting within the second-third decade of life. At the same time, cognitive deterioration worsens and produces in late disease stages frank dementia. The lower rate of progression observed in this study, when compared to previous analyses (Stevanin et al. 2008a), might relate to ethnic differences among patients or to a different clinical setting, being mostly pediatric in our study and more related to adult neurology centers in the previous one.

Table 4. Frequency of *SPG11* mutations according to the clinical categories outlined in the Methods.

Phenotypes	Group I: HSP + MR + TCC	Group II: HSP + TCC	Group III: HSP + MR, no TCC	Group IV: Apparently pure HSP	Sum
Number of index cases					
Familial	20	4	4	3	31
Sporadic	8	10	2	0	20
Mutation detected					
Familial	14/20[§] (70%)	1/4[§] (25%)	0/4	1/3 (33%)	16/31 (52%)
Sporadic	8/8 (100%)	1/10 (10%)	0/2	0	9/20 (45%)

[§]Refer to index cases in the families.

Neuroimaging appears even more helpful in deciding on performing genetic analyses. Thin rostral corpus callosum with a “beaked” shape and mostly sparing the splenium, frequently associated with "flames" at the frontal horns involving periventricular and deep cerebral white matter, with or without mild cerebral atrophy, appear the distinctive neuroradiological features of this disorder. In advanced disease stages, atrophy of higher cortical regions such as the prefrontal cortex matches thinning of the anterior corpus callosum (Figure 3). In a small set of individuals presenting without TCC, mutations in *SPG11* are found (8% in this study; 4.5% in Stevanin et al. 2008a) after a disease duration of less than 5 years. In such cases, however, either the presence of a sib with a full syndrome (clinical and neuroradiological) or evidence of learning disabilities and white matter lesions raise the clinical suspicion and we cannot exclude that follow-up MRI might show a “curved” shaped, thinner anterior corpus callosum.

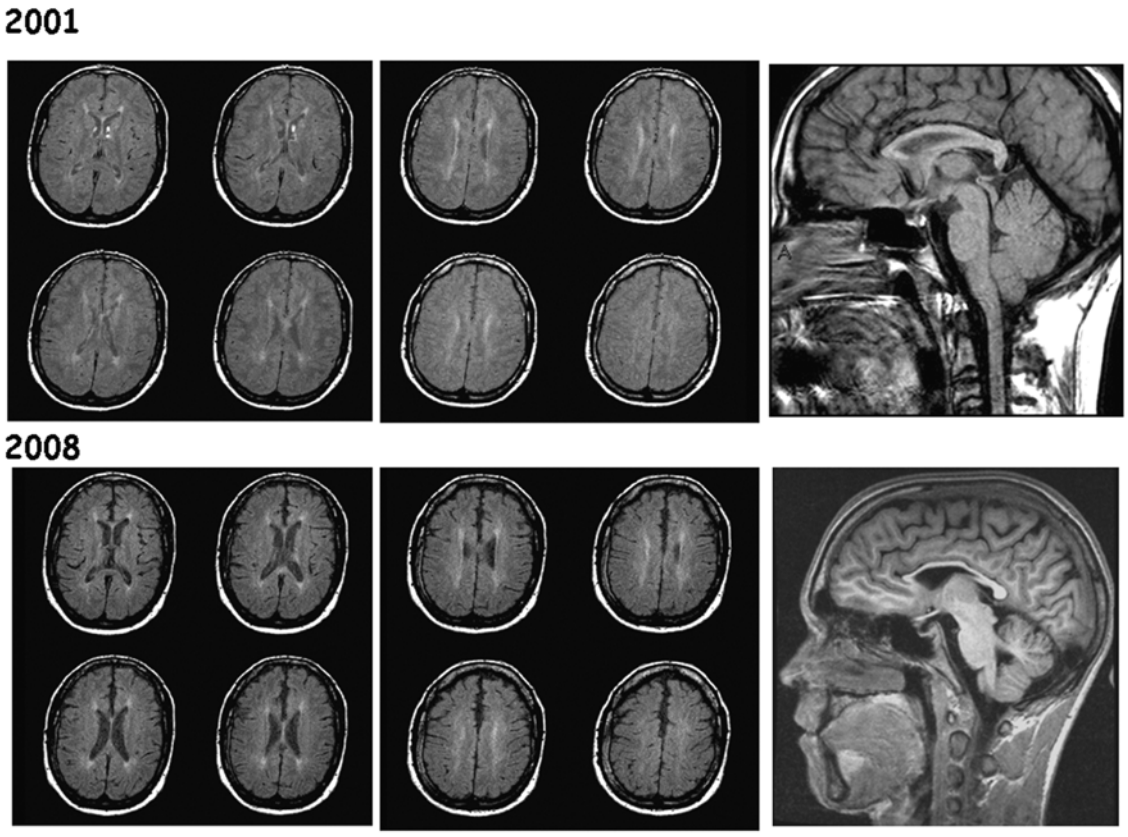

Figure 3. Axial and sagittal brain MRI images after a 7-year follow up in patient SP-H28 harboring the heterozygous p.F1752SfsX1837 and p.C1996LfsX1999 mutations in SPG11 showed worsening of TCC, “flames” at ventricular horns, and progression of cortical atrophy.

When clinical and neuroradiological features are combined, they also remain a useful criterion for deciding to screen isolated patients. In our cohort, 9/20 of cases (45%) with complicated HSP without a positive family history harbored mutations in *SPG11*. Eight patients had a combination of HSP+TCC and cognitive impairment (Table 4). This higher than expected positive mutation rate is different from what has been observed by others (1/25, 4%) (Paisan-Ruiz et al. 2008) but might depend on the use of more stringent selection criteria or might relate to different patients' ethnicities. In general, however, it is hard to speculate on the rate of *SPG11* mutations when patients under screening have less canonical phenotypes, such as HSP + mental retardation without TCC or pure spastic paraplegia with onset in early adulthood and no family history because we did not test enough patients meeting these criteria. Analyses in this latter group of patients, by far the most frequent in the clinical practice, require more cost-effective modalities of molecular screening after a more complete clinical examination and a dedicated *SPG11* testing.

About half of patients, both sporadic and familial, did not show mutation in *SPG11* notwithstanding our in depth mutation screening. The clinical presentation and the MRI features in such cases did not grossly differ from patients having both alleles mutated. These findings reinforce previous notions on genetic heterogeneity in ARHSP-TCC (Casali et al. 2004; Boukhris et al. 2008a). Although the presence of non-conventional variants remains possible, mutations in other genes are also to be expected. For instance, it has been shown that a subgroup of ARHSP-TCC with clinical features highly similar to *SPG11* cases (Elleuch et al.2007) are instead linked to the *SPG15* locus on chromosome 14q (about one in four in Boukhris et al. 2008a; Boukhris et al. 2008b) or mutated in the corresponding gene (Hanein et al. 2008). The future identification of other HSP-TCC related genes will permit better correlations with neuroimaging and clinical phenotype.

In summary, *SPG11* mutations appear the most frequent genetic determinant of autosomal recessive HSP worldwide with cases described on European, Asian and North-African genetic background. At least in Italy, *SPG11* seems to account equally for sporadic and familial cases and it currently denotes the most common etiology in ARHSP, with or without TCC, mirroring what *SPG4* represents for the autosomal dominant forms. Our results suggest that *SPG11* should be tested in patients, even with no family history, if progressive motor degeneration and early onset mental decline are associated with distinctive brain MRI since onset or after a few years of disease duration.

ACKNOWLEDGMENTS

The authors are grateful to the families and to the clinicians who referred patients to us. We thank Dr. C. Depienne for helpful discussion. The study was funded by the Agence Nationale pour la Recherche (France, to A.D. and G.S.), the Verum foundation (Germany, to A.B.), the Groupement d'Interet Scientifique – Institut des Maladies Rares (France, A04180DS/A04139DS to G.S.), the Association Strümpell-Lorrain (to the SPATAX network and A.Bo.), and PRIN-2006063820 (Italy, to A.M., A.F., and C. C.). Ga.S., An.M., M.M. acknowledge the financial support from the ISS (Istituto Superiore di Sanità) and IRCCS-Fondazione Don Gnocchi. P.S.D. and F.M.S. were supported by grants from the ISS, Fondazione Mariani ONLUS and Telethon Italy (GGP06188). The E-Rare EUROSPA network grant (to A.B. and F.M.S.) is also acknowledged.

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Supp. Table S1. Oligonucleotide primers and annealing temperatures (T-°C) used to amplify the 5'- and 3'-untranslated regions (UTR) of the *SPG11* gene and two novel intragenic dinucleotide markers.

Region flanking *SPG11*	FORWARD SEQUENCE (5'-3')	REVERSE SEQUENCE (5'-3')	T°C
5'UTR-1	CAGGCGTCAAAGAAAGCACT	TAGCAAAGCCACATCTGCC	60°
5'UTR-2	TAAGCTTAGCTGGGGCTGTG	CCTTGCCAACATAGGGAGAC	60°
5'UTR-3	AGGATAGCCTTCATTACAGGTTT	TTCTGAGGACAGAGATGACCA	60°
3'UTR	TGGATGAACAATCATCTAAAATCAA	TCATAAAACTTGTGTTCGTATGCC	60°
Intragenic *SPG11* markers			
chr15:42720928	GAATACATCCACCACACAAAC	GTGCTGGCAAGAACATCAAG	60°
chr15:42800751	CATATTATACCACACTTACC	TTTGGAAAGTCAAGGTGAGC	60°

Supp. Table S2. Oligonucleotide primers, annealing temperature (Ta-°C PCR), anticipated product size in base pairs (bp) and DHPLC conditions (T-°C DHPLC) used to analyze the 40 exons of the *SPG11* gene.

Exon	bp	Ta-°C PCR	5'- 3' Primers Forward/Reverse	T-°C DHPLC
1	308	60	agtcaggttccggcgaaagt /ccaactctccctcagcactt	54.8
2	323	62	accaggtcaactaaactgttctct / tatgctgaaagaccacctgtaga	55.3
3	305	58	ccagttgtaaaattgtgacc / tcaatcaacacttctaccac	55.1
4	320	62	gttaggcatacttacaaaactggc / cgaggatatttttaacctcttatca	54.8 - 52.8
5	330	60	caggagcagtagtaacacaa / aaagggtacagcgtcagcat	54.9
6	450	58	ctgtgacaggtgttaagtta / atctaatacaagacagtctc	54
7	275	58	tagtactgaagtattgagta / ttaagtaatgttcttgggca	50.6
8	450	60	cttgccccagattgcataat / tccaaaaagtacgtaaaatccca	54.1
9	342	62	gcaggtaataagcctgcagaa/ccccccttcctagctgctatt	54.1
10	331	62	cacacacacaaattggcaca / aacctttgccaaacattctga	54.1
11	293	57	gttacataaatgtataatccctg / cattttaagactttatggattac	54.6
12	210	62	tgttcaaaatagttccattacaaaa / tttcttccaaggttttcttcca	52.5
13	289	62	tttgcaaaagtgcttgatttt / tgcaggctcagttccacata	51.5
14	246	62	ggaatgatgccttttctcc / tctcacacttgccttctgga	55.8
15	345	60	cacagcgagatcctgtctca / cctcactgtaagatgatgccc	55-58
16	309	62	tgtgggcatgatttggtcta / acctgctcaaggacaaatgc	55.2
17	239	62	aatcatcgcctgagcaaaat / ccagtgactgatccaaagca	53.9
18	324	58	ccctcttaaggagaaaaacac / cagccttatcctctgctctt	53-50
19	299	60	gctaatcttgtttcacaagg / cctggctgaactctgataga	55
20	311	62	tggaaaaggggagcagacta / tgcgaactatttttcctttgg	52.3
21	303	58	ttccatgtgcaaatctgaaatta / tcccaaagtgctgggattac	58
22	383	62	gaggaggccacaaatcacat / gccttagacctcgtcacacc	55.8
23	356	62	tgctcaggttttgacttttctc / tttcactgatggcaagatgc	53.1
24	267	60	accacccccacctctaattc / ctacacaacagaaagaatgc	57.1
25	361	60	ccagctgaaactgaaagttgg / ctgggtacttacttcaggct	56.6
26	293	60	tgtacatttgccaggtaatcca / cttaagctctggaaagaagtg	56
27	330	62	cactgtgccctgccttatta / tgtgcctgagtaaccgagtg	53.6
28	329	62	tcccagatttggaggttttg / tgcattttaatttcctaactaccc	53.5
29	330	56	gctgtagtggcattttattg / cctgggtgacagagcaagac	56
30a	323	62	gaggtgggaggatctcttg / gatgtgttcagagcagccaa	58-55
30b	304	62	taagctggaggagctggaga / ttgttgtccccttaacttgg	58-55
31	318	62	cagggagcttcaagcagaga / tgacttggcaatgtccaaaa	56.9
32	323	60	cctggcttctaaaagtggcc / aagcacaacatccaaatcctt	58.8
33	349	62	agctgcagagctccataagc / taggcatccagagcaggaac	57.6
34	336	62	gaggttgacagtgggcagcca / gcccagccaactctcaagta	56.9
35	312	62	ggcatctgaaagcaaccact / ccctccattttcccaagagt	54
36	376	62	caacaggaaagcacacatgc / gtgtggctgtgacctcactc	52
37	313	62	aacatggctgggatgtttct / ttcctggttggcctatgatg	57.6
38	315	62	ggggtgaataccgttgtgag / acctctgggttccatgagtg	56.9
39	380	62	aatgccaaacacacacctga / ctcaaagcagaggcaaggag	53.2
40a	390	62	agactgctcctctgcactcc / ccgggattgttcaactttagc	54.4
40b	321	58	cagtatcttaacctgtacat / ccgggattgttcaactttagc	54.2